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(54) Title: COLD AND CRYOPRESERVATION SOLUTIONS AND METHODS FOR HUMAN TISSUE SLICES (57) Abstract Cold and cryopreservation solutions for tissue slices include a limited amount of glucose. The solutions provide extended cold storage of the slices without a loss of viability. The solutions also allow cryopreservation of the slices, so that the slices may be stored until needed and then thawed, cultured, packaged and distributed.		

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1 COLD AND CRYO-PRESERVATION SOLUTIONS
AND METHODS FOR HUMAN TISSUE SLICES

5 FIELD OF THE INVENTION

The present invention relates generally to methods and solutions for the preservation of tissue. More particularly, this invention relates to solutions for preserving tissue slices in a viable state and cold- and cryo-preservation methods for human tissue slices employing these solutions.

10

BACKGROUND OF THE INVENTION

Thousands of chemical substances are examined each year for their biotransformation and potential toxicity. Nearly all chemical toxicity tests will be conducted on rats, mice, guinea pigs, rabbits, cats, dogs and monkeys, with the results from these animal experiments being extrapolated to humans. While the reactions of rats, rabbits and mice to chemicals together with metabolite patterns are currently considered acceptable predictors of the toxic effects these substances will have on the human organism, the significance of animal experimentation and subsequent data extrapolation is a questionable science since species variation can be significant. By utilizing human *in vitro* biotransformation technology, the fate and disposition of potentially toxic, environmental/industrial chemicals or prospective drugs in man can be more accurately determined without exposing living humans or laboratory animals to the actual compounds.

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For the past several years, metabolism has been studied using subcellular (microsome) fractions obtained from human livers. Systems such as this are important for metabolite identification and rate of formation. The use of such subcellular fractions only allows one to determine if a particular enzymatic process exists within the fraction and its ability to react with a given compound. However, the observance of a specific enzymatic activity in an isolated subcellular fraction does not mean that this reaction will be expressed to the same extent *in vivo*. Extensive

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1 cellular organization and compartmentalization within the cell determine chemical
disposition of a test compound. Thus, it would be optimal to have an in vitro system
that retains the tissue's inherent organization and which will best reflect human in
5 vivo biotransformation. An intact in vitro metabolism system would also mimic both
the metabolic and chemical reactions that occur during in vivo metabolism.

Intact sections of human tissues appear to maintain crucial
characteristics of cellular organization while allowing the researcher the versatility of
an in vitro system. This is important since there is a heterogeneous distribution of
10 biotransformation enzymes within the tissue and intact tissue sections sample all of
these sites exactly as in the whole organism.

Human livers, kidneys, lungs, hearts and pancreas are harvested from
organ donors usually in a hospital or organ bank and made available through a
15 nationwide network the transplant centers. Occasionally, suitable matches can not be
found or the prospective recipient develops complications and the tissue then becomes
available for research. In order to do certain biological studies such as xenobiotics
metabolism, intermediary metabolism, hormone receptor studies or chemical induced
20 toxicity, the tissue has to be fully intact and comparable in its viability to the
standards set for transplantable tissue. This optimal viability is particularly important
for biochemical and cell biological studies requiring fully functional cells.

Two investigations, Pollard and Dutton, reported limited success using
25 tissue fragments and slices in drug disposition studies, but mentioned two specific
problems associated with their methods. The first was their inability to prepare
uniform, viable sections rapidly. The second problem centered around the failure to
maintain tissue viability throughout the experiment.

In the mid 1980's the first problem encountered by Pollard and Dutton
30 was overcome by the introduction of the Krumdieck et al. tissue slicer to produce
non-traumatized, precision-cut liver slices ($\pm 5\%$ variation in thickness) from 60 to
500 microns thick at a rapid rate under physiological conditions.

The problem of maintaining tissue viability during an experiment,
35 however, has not yet been satisfactorily overcome. Inappropriately cultured tissue

1 sections no longer have the gas, fluid and nutrient exchange that exist in vivo. To
overcome these deficiencies, an incubation system was developed in which the liver
slices were exposed to the gas phase while the side resting on the support was
5 constantly bathed in media. The media was supposed to coat the whole slice by
capillary action, and the submerged side receives gas via diffusion. This however,
proved inadequate to maintain the slice. The submerged slice rapidly deteriorated and
only cells in the gas-tissue interface remained viable. A number of investigators have
maintained slices in culture for 12 hours to several days with only the cells at the gas-
10 tissue phase remaining viable.

An organ culture system was developed in an attempt to overcome the
inadequate oxygenation of the conventional culture system. This new system
combined the advantages of increased surface area for oxygenation and nutrient
15 uptake with those of conventional organ culture (maintain organ architecture). Smith
et al. floated slices into cylindrical, stainless steel screen supports fitted with two
narrow stainless steel sleeves. The tissue adhered to the screens during culture. The
cylinders were then placed inside vials that contain sufficient media to wet the slices
20 when the vials are on their sides. The vials were flushed with 95:5% O₂:CO₂, sealed,
and incubated on a heated (37°C) vial rotator. As the vial was rotated the support
inside also rotated causing the underside of the tissue to be alternatively exposed to
media or gas while the upper side was continuously exposed to gas. This dynamic
25 organ culture system was far superior to conventional systems resulting in a
completely viable slice for up to 48 hours. In addition, it has been reported that
animal liver slices seemed to improve during culturing, raising the possibility that the
human liver slices might actually repair themselves during this type of culturing.

U.S. Patent No. 4,920,044 to Bretan describes an intracellular flush
30 solution for preserving organs. By way of background, Bretan provides the
compositions of four other flush solutions, namely: Collins-2 Flush; Sacks-2 Flush;
Belzer perfusate; and UW-1 Flush. Bretan states that D-glucose has been recently
shown to exacerbate acute renal ischemia damage in dogs. Mannitol is therefore
35 substituted for glucose in the Bretan solution (Bretan, column 10, lines 32-39).

1 U.S. Patent No. 4,798,824 to Belzer et al. describes perfusate for the
preservation of organs, particularly kidneys. The Belzer et al. '824 perfusate contains
hydroxyethyl starch ("HES") in place of human serum albumin for colloidal osmotic
5 support.

U.S. Patent No. 4,879,283 to Belzer et al. describes a solution which
includes hydroxyethyl starch ("HES") for the preservation of organs. Belzer et al.
indicate that glucose, the main impermeant in Collins' solution, is not an effective
impermeant for the liver or pancreas and readily enters cells. Thus, Belzer et al.
10 completely remove glucose from their formulation and replace glucose with HES.

Belzer et al. have also reported that preservation solutions containing
high concentrations of glucose pose a two-fold disadvantage since glucose is not an
effective impermeant to prevent cell swelling and glucose could stimulate acidosis.
15 Belzer et al., Transplantation, Vol. 45, pgs. 673-676, (April, 1988).

No prior cold preservation solution known to applicants has been
successful in maintaining the viability of tissue for longer than 72 hours.

It is an object of this invention to provide a preservation solution which
20 permits the cold preservation of viable tissue for periods longer than three days,
preferably for seven to ten days.

It is a further object of this invention to provide a method of cold
preserving tissue for up to ten days while maintaining viability of the tissue and for
cryopreserving tissue for extended periods up to three years while maintaining the
25 viability of the tissue.

It is also an object of this invention to expand the availability of human
tissue for testing, including xenobiotics metabolism, intermediary metabolism,
hormone receptor and chemical induced toxicology testing.
30

It is a further object of this invention to provide human tissue samples
for testing, which tissue samples preserve the cellular organization and
compartmentalization within the human tissue sample. It is a further object of the
present invention to provide an organ culture system which allows for the accurate
35 determination of in vivo biotransformation with an in vitro system.

1 It is also an object of this invention to provide a method of freezing and
storing viable human tissues. It is a further object of this invention to provide an
integrated procedure which includes tissue slicing - cold storage - cryopreservation -
5 special packaging for shipping and later culturing - cold storage during transport and
packaging in specified self-righting cold storage containers. All steps may
advantageously be performed in one basic solution, which is modified by one
additional ingredient (DMSO) for cryopreservation and replacement of some
10 potassium for sodium in the final culturing phase. It is also an object of this
invention to provide a preservation method which will allow the establishment of a
tissue bank comprised of valuable human tissue slices to be utilized for research.

 It is a further object of this invention to reduce the amount of
preclinical animal trials by providing a method for the preservation and distribution of
15 viable human tissue slices for testing.

SUMMARY OF THE INVENTION

 Preservation solutions in accordance with the present invention contain
20 glucose in an amount sufficient to maintain the metabolic functions of the tissue cells
but insufficient to stimulate acidosis and permit the cold preservation of viable tissue
for periods longer than 72 hours, normally up to about ten days. The preservation
solutions of the present invention are suitable for use in conjunction with a
25 cryoprotectant to provide the cryopreservation of viable tissue for periods of three
years or longer.

 The present invention also embraces methods of cold preservation
comprising the steps of contacting a tissue slice with a preservation solution
containing glucose in an amount sufficient to allow the metabolic functions of the
30 tissue cells but insufficient to result in acidosis and storing the slices at about zero
degrees centigrade.

 In another aspect the present invention embraces a method of
cryopreserving tissue slices comprising the steps of: contacting the tissue with a
35 preservation solution containing a) glucose in an amount sufficient to maintain the

- 1 metabolic functions of the cell but insufficient to cause acidosis and b) a
cryoprotectant; and then cryopreserving the tissue by cooling at a controlled rate
whereby the viability of the tissue slices is maintained in the cryopreserved state.
- 5 After a period of months or years, the tissue may be thawed, reconditioned,
specifically packaged for dynamic culture, placed in the preservation solution and
stored at about zero degrees centigrade.

Tissue slices which have been contacted with the preservation solutions
of the present invention may be packaged and shipped in self-righting cold packs in
10 accordance with another aspect of this invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

The preservation solutions of the present invention contain glucose in
15 an amount sufficient to maintain the metabolic function of the cell but an amount
insufficient to cause acidosis. The effectiveness of any physiologically acceptable
preservation buffer solution may be improved by adjusting the amount of glucose in
the solution in accordance with the present invention.

20 The preservation solutions of this invention may be based upon any
physiologically acceptable solution. By the phrase "physiologically acceptable"
solution it is meant that the solution tends to (1) minimize hypothermic-induced cell
swelling; (2) prevent intracellular acidosis; (3) prevent the expansion of extracellular
space; (4) prevent injury from oxygen free radicals; and (5) provide substrates for
25 regenerating high energy phosphate compounds.

In general, a physiologically acceptable solution will contain an
effective impermeant to prevent or minimize hypothermic induced cell swelling.
Impermeants which may be included in the physiologically acceptable solution
30 include, but are not limited to K^+ - lactobionate, raffinose, hydroxyethyl starch,
gluconate (K^+ salt), saccharate (K^+ salt), mannitol and polyvinylpyrrolidone.

Physiologically acceptable solutions also generally include a hydrogen
ion buffer, such as for example, a phosphate. Adenosine, an ATP precursor or other
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1 agents which provide precursors for the regeneration of high energy phosphate
compounds may also be included in suitable base solutions.

5 Additionally, the base solution may include substances that create
colloidal osmotic pressure and allow the free exchange of constituents of the solution
without expanding the interstitial space. Suitable ingredients for these purposes
include albumin, polyvinylpyrrolidene, polyethylene glycol, dextran, and hydroxyethyl
starch.

10 Ingredients or agents which inhibit the generation of O_2 free radicals
may also be included in the physiologically acceptable base solution used to form the
solution. Oxygen free radical generation is believed to be inhibited by allopurinol, an
inhibitor of xanthine oxidase as well as mannitol and other radical scavengers and
heavy metal complexes such as deferoxamin and EDTA.

15 Other ingredients which may be included in a physiologically
acceptable base solution include, but are not limited to inorganic salts such as KCl,
MgSO₄, MgCl, NaHCO₃, KHCO₃, reducing agents such as glutathione, vitamin E,
vitamin C, hormones such as dexamethasone, insulin, antibiotics such as penicillin,
20 gentamycin and bactrim or calcium channel blockers such as verapamil.

Suitable preservation solutions may have an osmolality in the range of
300 to 450 and a neutral to slightly basic pH, preferably a pH in the range of 7.4 to
7.6.

25 Presently known physiologically acceptable solutions upon which the
solutions of the present invention may be based include those disclosed in the above
mentioned U.S. Patent Nos. 4,920,044; 4,798,824; and 4,879,783 the disclosures of
which are incorporated herein by reference. The compositions of some of the
presently known solutions are presented in Table 1. These known solutions may
30 serve as the base for the preservation solutions of the present invention, provided their
glucose content is adjusted to an amount which is sufficient to maintain the metabolic
functions of the cell but insufficient to cause acidosis within the cell. By adjusting the
glucose content of these known solutions their ability to preserve tissue slices can be

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- 1 substantially improved. Use of the preferred solutions of this invention may provide a two to three-fold increase in preservation time over known preservation solutions.

TABLE 1
COMPOSITION OF DIFFERENT INTRACELLULAR RENEVAL FLUSH SOLUTIONS(g/L)

	COLLINS-2 FLUSH	SACKS-2 FLUSH	BELZER PERFUSATE	UW-1 FLUSH	BRETAN
KH_2PO_4	2.0	4.16	3.4	3.4	1.54-2.56
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	9.70	9.70	--	--	7.38-12.2
KCl	1.12	--	--	--	0.84-1.40
KHCO_3	--	2.30	--	--	0-2.87
Mannitol	--	37.5	--	--	20-37
Glucose	25	--	1.5	--	--
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	7.38	--	8	1.2	0-4.62
MgCl_2	--	(2 meq/ml)	--	--	0-.4meq/ml
Adenosine	--	--	1.3	1.34	0.75-1.25
Sodium Glutathione	--	--	17.5	0.92	--
Albumin	--	--	5.3	--	--
NaHCO_3	0.84	1.26	--	--	0.63-1.05
Allopurinol	--	--	0.113	0.113	0-0.125
Verapamil	--	--	--	--	0-1.0
K^+ Lactobionate	--	--	--	39.8	--
Raffinose	--	--	--	17.8	--
Hydroxyethyl Starch	--	--	--	5-	--
Osmolality (mOsm/kg)	320	430	300	320-330	255-425
pH	7.00	7.00	7.10	7.40	7.20-7.50

SUBSTITUTE SHEET

1 While the amount of glucose employed in the solutions of the present
invention will vary somewhat depending upon the type of tissue being preserved and
the composition of the base solution, typically the amount of glucose employed will
5 be below about 1000 milligrams per liter and above about 20 milligrams per liter
(between about 5.55 mM and about 0.17 mM of glucose). For liver tissue, the
amount of glucose should normally be below in the range 500-750 mg per liter. For
kidney tissue the amount of glucose should normally be in the range 750 mg - 1000
10 mg/Liter. For lung tissue the amount of glucose used should normally be in the
range 300 mg - 600 mg/Liter. For heart tissue, 600 mg - 900 mg/Liter glucose
should normally be used. For pancreas tissue, 300 mg - 500 mg/Liter will normally
be appropriate.

The amount of glucose present should be an amount sufficient to
15 maintain the metabolic functions of the cell. Any known method may be used to
determine if a sufficient amount of glucose is present. For example, one test for
determining that glucose is present in an amount sufficient to maintain the metabolic
functions of the tissue cells would be to monitor the consumption of glucose and/or
20 the generation of metabolic products of glucose, such as, for example, lactic acid.
Experimental protocols to determine the presence of the appropriate amount of
glucose may be readily designed by those skilled in the art.

Likewise, any known method may be used to determine if an excess of
25 glucose is present. Typically, an excess of glucose will result in increased synthesis
of lactic acid and an increase in the concentration of hydrogen ions. Tissue acidosis
can damage cells and induce lysosomal instability, activate lysosomal enzymes and alter
mitochondrial properties. Thus, any assay which would indicate the presence of any
of these effects may be used to identify an excess of glucose.

30 Perhaps the simplest method of determining whether glucose is present
in an amount sufficient to maintain the metabolic function of the cells but insufficient
to cause acidosis is to prepare a series of solutions which differ only in their glucose
content within the limits set forth above and then experimentally determine which
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1 glucose concentration provides the optimum cold storage time for a particular tissue type.

The preservation solutions may be prepared in any known manner.

5 Typically, the ingredients are simply added to distilled or deionized water. Particular orders of addition may be preferred in certain circumstances. For example, it may be preferred to delay adding any component which is not stable for long periods in an aqueous environment, such as, for example, magnesium ions, reduced glutathione or
10 certain antibiotics. In that case, all other ingredients may be mixed together, with the sensitive materials being added just prior to use of the solution. It should also be understood that the solution may be provided as a concentrate to be diluted with distilled or deionized water just prior to use.

A list of the ingredients of the preferred preservation buffer solutions is
15 presented in Table 2. A range for the amount of each ingredient in the preferred solutions as well as the exact formulation of the most preferred solution are also provided. The most preferred solution is hereinafter referred to as "V-7 solution".

Special features of this most preferred, V-7 solution are that it:

- 20
- 1) is hyperosmolar as compared to extracellular fluid;
 - 2) is high in its potassium content, approximating an intracellular potassium content;
 - 3) is devoid of calcium;
 - 25 4) is rich in magnesium as compared to extra or intracellular fluid;
 - 5) contains nonpermeable anions which are not found in any other cold storage solutions;
 - 6) contains small amounts of ascorbic acid;
 - 7) contains a number of acid generating substrates such as
30 succinate, fructose and glucose;
 - 8) contains important anions for general cell function such as chloride and bicarbonate;
 - 9) is free of plasma expanders and other high molecular weight
35 compounds;

- 10) contains complexing agents for iron and other heavy metal ions.

Slices kept in V-7 solution under the oxygen tension of ambient air at the temperature of melting ice (0°C) maintain their ability to express full viability (when cultured at 37°C) for at least three days and up to ten days. The time of maintenance is somewhat dependent on the tissue and is highest for kidney and lowest for heart.

TABLE 2

V-7 SOLUTION

	<u>Preferred Range</u>	<u>Most Preferred Composition</u>
Gluconate (K salt)	30-120mM	60mM
Saccharate (K salt)	30-120mM	60mM
Potassium phosphate (monobasic)	25-30mM	25mM
Sodium succinate	1-3mM	2mM
Magnesium sulfate	3-6mM	5mM
Magnesium chloride	1-3mM	2mM
Potassium bicarbonate	1-3mM	2mM
Glucose	2-4mM	2mM
Fructose	2-4mM	2mM
Glutathione (reduced)	3-6mM	6mM
Ascorbic acid (K salt)	2-10mM	5mM
Adenosine	3-5mM	5mM
Antibiotics (gentamycin or penicillin)		μmolar amounts
Deferoxamine mesylate		μmolar amounts
pH	7.3-7.5	7.4
Osmolality	320-360	340

1 While further process aspects of the invention will be described specifically with respect to the V-7 solution, it should be understood that any preservation solution in accordance with the invention may be employed.

5 The preservation solutions of this invention may be used to preserve a variety of types of tissue slices. The tissue slices may be prepared in any known manner and preferably range from about 150 to about 250 μm in thickness to allow for optimal nutrient and gas exchanges between the slice, media and atmosphere.

10 In the preferred tissue slice preparation method a mechanical precision cut tissue slicer having a motor-driven oscillating blade rapidly produces slices of nearly identical dimensions in a controlled environment with minimal tissue trauma. Fresh human tissue is placed in ice cold V-7 solution and cylindrical tissue cores are prepared by advancing a rotating sharpened metal tube with constant pressure into the
15 tissue which is suitably positioned on a support on the table of a drill press. Following the preparation of several uniform cylindrical tissue cores, tissue slices of defined thicknesses are obtained by use of the mechanical slicer which operates while submerged under ice cold V-7 solution. Individual tissue cores are placed into a
20 matching cylindrical plastic holder and are lightly compressed with a piston holding adjustable weights. The combination of the distance between an adjustable base plate on the bottom of the tissue holder and the weight of the piston above determines the thickness of the slices. Slices are produced by pulling the immobilized weighted
25 tissue cylinder across the rapidly oscillating razor blade. The freshly sectioned slices are then swept away by a stream of V-7 solution and collected in a basket. Slices can be produced at a rate of one per second. Upon completion of the slicing process, slices are pooled in a flat plastic tray and then transferred to their final culture
30 vessels. In a preferred embodiment multiple cores are cut simultaneously such that the highest rate of slice production is achieved.

 A common problem with conventional static organ culture techniques is degeneration of cultured slices at the slice-media interface or in the center of the slice. In the preferred culture system of the invention, however, both the upper and lower
35 surfaces of the cultured tissue are exposed alternatively to the gas and liquid phase

1 during the course of incubation. This is different from the method described by
Smith et al. and can only be achieved with the slices affixed to the screens. The
slices are affixed to the screens by applying a carrier in the form of a thin film of a
5 non-toxic natural or synthetic material which is permeable to gas and the culture
medium. Suitable film-forming materials include agarose and gelatin. In a preferred
embodiment, individual tissue slices are floated from a tray containing a 0.5%
agarose in a balanced salt solution at 37°C into small stainless steel mesh half
10 cylinders. These half cylinders are rolled from rectangular pieces of stainless steel
screen and are held in teflon/silicone rubber cradles. Following the loading of a
slice, the cradle is removed from the loading tray, blotted and cooled to produce a
thin agarose gel film around the slice and screen which solidly anchors the slice onto
the screen. Cradles with the slices affixed to the screen are then immersed into V-7
15 solution and sent to outside laboratories where any desired testing may be conducted
on the slices. Alternatively, the cradles may be placed horizontally onto a
temperature controlled vial rotator and cultured in a culture medium such that every
time the cradle rotates it will dip the affixed slice through the surface of the culture
20 medium, thus exposing both sides of the slice to the media and gas phase. Suitable
rates of rotation are between about 1 and about 4 revolutions per minute. The
preferred culture medium is a modified V-7 solution, particularly a V-7 solution in
which the majority of the potassium ions are replaced by sodium ions. Most
preferably, between about 85 and about 95% of the potassium ions present in the V-7
25 solution are replaced by sodium ions.

Several experimental protocols for determining slice viability or
metabolism are known to those skilled in the art, including slice K^+ content, protein
synthesis assays (for example wherein the incorporation of 3H -leucine in precipitated
30 proteins is measured), lactate dehydrogenase leakage (diagnostic kit available from
Sigma Chemical Co., St. Louis, Missouri), total protein measurement (diagnostic kit
available from Pierce Chemical Co., Rockford, Illinois). and xenobiotics metabolism
measurement (see Wishnies et al. (1991), *Cryobiology*, 28:216-226). The particular
35 protocol selected is a matter of convenience.

1 In the examples that follow, K^+ content of the slices was determined as
follows: slices were removed from the scintillation vials, blotted, weighed, and
placed into 1 ml of distilled water. Slices were then homogenized by sonication with
5 a cell disrupter at an intermediate setting (Model 350, Branson Sonic Power Co.,
Danbury, CT). The homogenate was centrifuged after the addition of 20 μ l of 70%
perchloric acid and the supernatant fraction assayed for K^+ by flame photometry
(Model CA-51 Perkin Elmer, Danbury, CT.). Results are expressed as μ moles K^+ /g
10 slice wet weight or as percent of control. See Fisher et al., In Vitro Toxicology
3:181-194) (1990). K^+ content of viable cells vary depending upon the particular
type of tissue involved. Typically, the K^+ content of viable tissue varies from about
50 to about 80 μ moles per gram of slice wet weight. A decrease in the K^+ content
indicates that cellular function is ceasing and the tissue is losing viability. The
15 percentage of K^+ lost generally reflects the degree of which cell function has ceased
and the tissue has died.

EXAMPLE 1 (COMPARATIVE)

20 Slices from three human livers were placed in a known preservation
solution of the type described by Belzer and commonly referred to as "U.W. solution"
and maintained at 0°C. The composition of the U.W. solution is given in Table I
above. The K^+ content of the slices was measured at various intervals as a measure
25 of the viability of the tissue. The results are shown in Table 3.

EXAMPLE 2

30 The solution used in Example 2 was the same as the U.W. solution of
Example 1, with the exception that 360 mg/L of glucose was added to the solution.
Again, slices from the three human livers were placed in the glucose-modified U.W.
solution and stored at 0°C. The results are shown in Table 3.

35

1 EXAMPLE 3

Slices from three human livers were placed in V-7 solution (See Table 2) and stored at 0°C. The results are given in Table 3.

5 The results for Examples 1-3 are given in Table 3 as an average of the values obtained plus or minus the standard error of the mean. The experiment was started at 0 time, viability measured at that point and compared with several solutions for various periods of time. The results of Examples 1 and 2 clearly demonstrate that the addition of a limited amount of glucose to known preservation solutions increases
10 the duration of viability of the tissue slice. A comparison of the K⁺ content of the slices between 48 and 96 hours shows a significant increase in viability is provided by an amount of glucose sufficient to maintain the metabolic functions of the tissue cells but insufficient to stimulate acidosis.

15

TABLE 3

20

	Hours	<u>EXAMPLE 1</u>	<u>EXAMPLE 2</u>	<u>EXAMPLE 3</u>
		U.W. SOLUTION	U.W. + GLUCOSE	V-7
25	0	66.7 ± 2.8	66.7 ± 2.8	66.7 ± 2.8
	24	45.8 ± 1.5	46.3 ± 1.0	65.0 ± 2.1
	48	33.7 ± 2.1	44.1 ± 1.7	63.2 ± 1.8
	72	30.8 ± 2.7	42.8 ± 1.8	63.7 ± 1.5
	96	30.1 ± 1.3	40.1 ± 2.1	62.9 ± 2.1
30	120	30.0 ± 1.0	35.7 ± 2.5	64.6 ± 2.5

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1 EXAMPLES 4-9

5 A series of preservation solutions having a range of glucose contents were prepared based on the above-described V-7 solution. The composition of each solution is given in Table 4. Slices of human liver were placed into contact with each solution and maintained at 0°C for ten days. The viability of the cells were then determined by measuring slice K⁺ content. The results are given in Table 4. The K⁺ content of a human liver slice at time zero is given in Table 4 as a control.

10 TABLE 4

15	<u>Example Number</u>	<u>Solution Composition</u>	<u>K⁺ Content (μmoles K⁺/gm)</u>
	Control	Fresh Slices	61.7
	4	V-7 without glucose	36.7
	5	V-7 with 1.0 mM glucose	44.0
20	6	V-7 with 2.0 mM glucose	59.8
	7	V-7 with 4.0 mM glucose	60.4
	8	V-7 with 5.0 mM glucose	43.1
	9	V-7 with 6.0 mM glucose	32.7

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30 The data presented in Table 4 show that for a preservation buffer solution having the composition of V-7, a glucose content of between about 2 and about 4 mM is optimal. V-7 without glucose was unacceptable since the amount of glucose present (i.e., none) was clearly insufficient to maintain the metabolic functions of the cells. V-7 with 6.0 mM glucose was also unsatisfactory since 6.0 mM glucose clearly exceeded the amount required to maintain the metabolic functions of the cell and resulted in acidosis within the cell. The appropriate optimal glucose

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1 content for other preservation solutions and tissues can likewise be determined in this
manner.

5 The preservation solutions of the present invention may also be used in
cryopreservation of tissue by adding a cryoprotectant to the V-7 solution. Any known
cryoprotectant can be employed. Suitable cryoprotectants include but are not limited
to, dimethyl sulfoxide, glycerol, ethylene glycol and propanediol. While the amount
of cryoprotectant added will depend upon the particular cryoprotectant chosen,
10 typically between about 5% and about 50% may be added to the cold preservation
solution employed.

In accordance with the cryopreservation method of this invention, tissue
slices are contacted with a mixture of V-7 solution and a cryoprotectant and then
cryopreserved by first cooling at a controlled rate, and then submerging the samples
15 in a cryogenic fluid. The particular rate of cooling will depend upon the type of
tissue being cryopreserved. For example, for human liver slices, a cooling rate in the
range 0.25 to 1.0 °C/min. may be used, while for kidney slices a rate in the range 10
to 15°C/min. may be used. Liquid nitrogen is the preferred cryogenic fluid.

20 In one preferred embodiment of the cryopreservation method of the
present invention, tissue slices are placed into a mixture of V-7 solution and dimethyl
sulfoxide ("DMSO"). Preferably, the V-7 solution is modified by adding between
about 8% and about 15% DMSO. The slices are maintained in the solution for 15-20
minutes at 0-20°C and then cooled at rates between about 0.25 and 1.0°C per minute
25 to -70°C. The slices are then rapidly immersed into liquid nitrogen at
-196°C. The slices can be kept in this condition for a period of time which can range
from months to years.

30 When there is a demand for the tissue slices, they may be retrieved and
warmed. In a preferred embodiment the slices are warmed to between -150 and
-100°C by placing them into the gaseous phase of liquid nitrogen for approximately 2-
5 minutes. Then the temperature is raised to -70 to -50°C for another 2-5 minutes.
The slices are then quickly thawed by immersing the slices in a large volume of 35-
35 39°C V-7 solution. At that point slices may be reconditioned by culturing them at

1 37°C in dynamic organ culture using fetal bovine serum as the reconditioning fluid or
alternatively using a V-7 solution in which 85-95% of the potassium ions are replaced
by sodium ions. This reconditioning fluid based on the V-7 solution may be prepared
5 by substituting sodium salts in the amounts given in Table 2 above for the
corresponding potassium salts contained in the V-7 solution. Preferably, the
potassium salt of saccharate is still included in this reconditioning form of V-7
solution. After 2 to 24 hours in the reconditioning solution, the slices are ready for
organ culturing which may be carried out in any commercially available culture
10 medium, or the above-mentioned reconditioning fluids. The final culturing may be
conducted at temperatures up to 38°C for up to 72 hours.

In another embodiment of the cryopreservation methods of this
invention, the tissue slices are placed in V-7 solution which has been modified by
15 adding up to about 45% propylene glycol as the cryoprotectant. The careful
introduction of these high concentrations of cryoprotectant is preferably done in a
series of steps of 5-10% at 0°C. Slices are maintained at each concentration step for
3-5 minutes and then transferred to the next higher concentration. The exact number
20 of steps will depend upon the final concentration of cryoprotectant to be used and the
particular cryoprotectant employed. The overall cold equilibration with the
cryoprotectant modified V-7 solution should normally not exceed 12 to 20 minutes.
Slices may then be loaded into low mass plastic sieves and extremely rapidly cooled
($> -10000^{\circ}\text{C}/\text{minute}$) by vertical immersion of the carrier sieves into liquid
25 nitrogen. The slices cryopreserved in this fashion should be translucent and glass-like
and not white and snowy. There should also not be any white spots on the
supercooled slices. Again the tissue is banked under liquid nitrogen in this state and
kept there until needed. At that time the tissue may be thawed at extremely rapid
30 rates ($> +10000^{\circ}\text{C}/\text{minute}$) in order not to pass through a stage in which ice crystals
are formed. Properly thawed slices can be reconditioned as previously described and
then reintroduced to V-7 solution for packaging and cold shipment.

As with any human tissue sample, a certain risk of contamination with
35 a variety of tissue borne disease causing agents exists. The tissue slices may

1 therefore be packaged in accordance with a further aspect of this invention in a
manner to minimize the risk of infection. The slices are packaged in accordance with
the invention such that they are enveloped in a suitable carrier and inserted into
5 disposable dynamic organ culture vessels. The main risk for anyone working with
this tissue would be direct blood contact as per a cut or prick with a sharp instrument
or through an open wound. Packaging in accordance with the present invention limits
exposure to the tissue by the researcher to the potential infectious agents borne by the
tissue. The protection by the present packaging is mainly due to the absence of any
10 requirement for sharp or pointed tools in the manipulation of such packaged slices.
Risks from additional aerosol exposure and manual handling can be essentially
excluded by proper handling precautions, such as for example double gloving,
wearing aerosol masks, and eye protection and working inside a bio-safety hood.

15 Packaging of slices in disposable dynamic organ culture vessels can be achieved by
using a special carrier material to affix the slices to a cradle. Suitable carrier
materials include non-toxic natural or synthetic materials capable of forming a film
which is permeable by both gas and the culture medium. Preferably, the carrier
20 material is applied by introducing the carrier material to a balanced salt solution.
Most preferably the carrier material will be liquid at 37°C but will form gels at lower
temperatures and will not melt if exposed once again to 37°C. Such carrier solutions
include but are not limited to 0.5% agarose and 2% gelatin solutions. When tissue
slices are loaded onto the cradles while submerged in warm (37°C) balanced salt
25 solution containing 0.5% agarose, blotted and cooled to lower temperatures (0-10°C),
a thin gel film will surround and attach the slice to the screen. After slices are
affixed to the screen, it requires vigorous shaking to remove them thus now making it
possible to wash medium over the upper surface of the affixed slice during dynamic
30 organ culture. The goal of alternatively exposing both upper and lower surfaces of
the slice to either gas or media phase has now been accomplished. Affixing slices to
the screens also helps in shipping cold preserved slices in V-7 to other laboratories.

Packaging tissue slices in the above manner also requires special
35 shipping arrangements such that the dynamic organ culture vessels bearing the

1 envelope with human tissue is at all times kept in a submerged position in V-7
solution. A special cold shipping container is contemplated in accordance with a
further aspect of the invention. The shipping container of the present invention
5 includes an inner container having a substantially spherical inner surface. The inner
container may simply be a spherical container such as a plastic globe. The spherical
container is placed into an outer receptacle. Preferably the outer receptacle is regular
in shape so as to allow stacking thereof and includes a recess adapted to receive the
spherical container. The outer container may advantageously be constructed as two
10 halves each having a hemi-spherical recess. Alternatively, the outer container may
have a cylindrical recess adapted to snugly receive the spherical container.
Preferably, for use in connection with the transport of tissue slices, the outer
container has insulating properties. To this end, the outer package may be molded
15 from expandable polystyrene beads.

Within the inner container is a self-righting member. The self-righting
member includes a riding surface which is substantially parallel to the inner surface of
the inner container. A lubricating material is located between the riding surface of
20 the self-righting member and the inner surface of the inner container. The lubricating
material may be any liquid which allows the self-righting member to slide smoothly
on the interior surface of the spherical container.

For cold shipping of tissue slices the self-righting member is preferably
25 a hemi-spherical block of ice and the lubricating material is water which is either
added to the container or produced by melting of the ice. The actual rack in which
the tissue loaded dynamic organ culture vessels are held is anchored tightly to this
half spherical ice block with appropriate anchors. This ice block and sample rack will
always assume a horizontal orientation because the ice block will float on its own
30 melted ice. This will allow the slices to be in the same relative orientation and
exposed to the V-7 solution at all times. When this special shipping container is
combined with slice affixment to the carrier screens in the organ culture vessel as
described above, slices will be continuously exposed to V-7 cold preservation solution
35 even when occasionally roughly handled during shipment.

1 WHAT IS CLAIMED IS:

1. A method of cold preservation and cold storage of thin human tissue slices comprising:

5 contacting the tissue with a physiologically acceptable preservation buffer solution containing:

a) one or more agents for preventing or minimizing hypothermic induced cell swelling;

10 b) one or more agents for providing nutrition, said nutritional agent being present in an amount insufficient to cause intracellular acidosis;

c) one or more agents for providing protection against injury from oxygen free radicals; and

15 d) one or more agents for providing precursors for the regeneration of high energy phosphate compounds.

2. A method as in claim 1 wherein said agents for providing nutrition and prevent intracellular acidosis comprise:

glucose at concentrations of about 2 mM to 4 mM;

fructose at concentrations of about 2 mM to 4 mM; and

succinate at concentrations of about 1 mM to 3 mM.

25 3. A method as in claim 1 wherein said agents for preventing or minimizing hypothermic induced cell swelling comprise:

gluconate at concentrations of about 30 mM to 120 mM; and

30 saccharic acid at concentrations of about 30 mM to 120 mM.

4. A method as in claim 1 wherein said agents for preventing injury from oxygen free radicals comprise:

35 glutathione at concentrations of about 3 mM to 6 mM;

1 ascorbic acid concentrations of about 2 mM to 10 mM; and
 deferoxamine mesylate at concentrations from about 5 μ M to 15
 μ M.

5 5. A method as in claim 1 wherein said agents for providing
 precursors for the regeneration of high energy phosphate compounds comprise:
 adenosine at concentrations of 3 mM to 5 mM.

10 6. A method as in claim 1 wherein the composition of said
 preservation buffer solution comprise:
 from about 30 to about 120 mM gluconate;
 from about 30 to about 120 mM saccharate;
 from about 25 to about 30 mM phosphate;
15 from about 1 to about 3 mM sodium succinate;
 from about 3 to about 6 mM magnesium sulfate;
 from about 1 to about 3 mM magnesium chloride;
 from about 1 to about 3 mM potassium bicarbonate;
20 from about 2 to about 4 mM glucose;
 from about 2 to about 4 mM fructose;
 from about 3 to about 6 mM glutathione;
 from about 2 to about 10 mM ascorbic acid;
25 from about 3 to about 5 mM adenosine; and
 micromolar amounts of antibiotics and deferoxamine mesylate.

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- 1 7. A method of storing tissue comprising:
 contacting the tissue with a physiologically acceptable
 preservation buffer solution containing glucose, said glucose being present in an
 amount sufficient to maintain the metabolic function of the cell but an amount
5 insufficient to cause acidosis; and
 storing the tissue at around 0°C.
- 10 8. A method as in claim 7 wherein said glucose is present in an
 amount from about 20 milligrams per liter to about 1000 milligrams per liter.
- 15 9. A solution for the preservation and storage of tissue comprising:
 a physiologically acceptable preservation solution containing--
 glucose in an amount sufficient to maintain the metabolic function of the tissue cells
 but an amount insufficient to cause acidosis.
- 20 10. A solution as in claim 9 wherein glucose is present in an amount
 from about 20 to about 1000 milligrams per liter.
- 25 11. A method of cryopreserving tissue slices comprising:
 contacting the tissue slices with a solution comprising a
 cryoprotectant and a physiologically acceptable preservation solution containing
 glucose in an amount sufficient to maintain the metabolic functions of the cells of the
 tissue but insufficient to cause acidosis; and
 immersing the tissue in a cryogenic fluid.
- 30 12. A method as in claim 11 wherein said glucose is present in an
 amount between about 20 milligrams per liter and about 1000 milligrams per liter.

13. A method of cryopreservation of thin human tissue slices
1 comprising:
contacting tissue with a physiologically acceptable
cryopreservation buffer solution comprising:
5 a) one or more agents for preventing or minimizing
hypothermic induced cell swelling;
b) one or more agents for providing nutrition but preventing
intracellular acidosis;
10 c) one or more agents for providing protection against
injury from oxygen free radicals; and
d) one or more agents for providing precursors for the
regeneration of high energy phosphate compounds;
15 e) a cryoprotectant.

14. A method solution as in claim 13 wherein said cryoprotectant is
selected from the group consisting of dimethyl sulfoxide, glycerol, ethylene glycol
and propanediol.
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15. A method as in claim 13 wherein said agents for providing
nutrition and prevent intracellular acidosis comprise:
glucose at concentrations of about 2 mM to 4 mM;
25 fructose at concentrations of about 2 mM to 4 mM; and
succinate at concentrations of about 1 mM to 3 mM.

16. A method as in claim 13 wherein said agents for preventing or
30 minimizing hypothermic induced cell swelling comprise:
gluconate at concentrations of about 30 mM to 120 mM; and
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saccharic acid at concentrations of about 30 mM to 120 mM.

17. A method as in claim 13 wherein said agents for preventing injury from oxygen free radicals comprise:

glutathione at concentrations of about 3 mM to 6 mM;
ascorbic acid at concentrations of about 2 mM to 10 mM; and
deferoxamine mesylate at concentrations from 5 μ M to 15 μ M.

18. A method as in claim 13 wherein said agents for providing precursors for the regeneration of high energy phosphate compounds comprise:

adenosine at concentrations of about 3 mM to 5 mM.

19. A method as in claim 13 wherein the composition of said cryopreservation buffer solution comprises:

from about 30 to about 120 mM gluconate;
from about 30 to about 120 mM saccharate;
from about 25 to about 30 mM phosphate;
from about 1 to about 3 mM sodium succinate;
from about 3 to about 6 mM magnesium sulfate;
from about 1 to about 3 mM magnesium chloride;
from about 1 to about 3 mM potassium bicarbonate;
from about 2 to about 4 mM glucose;
from about 2 to about 4 mM fructose;
from about 3 to about 6 mM glutathione;
from about 2 to about 10 mM ascorbic acid;
from about 3 to about 5 mM adenosine; and

1 from about 5% to 50% v/v dimethylsulfoxide micromolar
amounts of antibiotics and deferoxamine mesylate.

20. A method as in claim 13 further comprising the step of cooling
5 the tissue at a controlled rate prior to immersing the tissue in the cryoprotectant.

21. A method as in claim 20 wherein the tissue is cooled at a
controlled rate to about -70°C .

10 22. A method as in claim 20 wherein said cooling is at a rate of
between about 0.25°C to about 12°C per minute.

15 23. A method as in claim 21 wherein said tissue is human liver or
pancreas slices and said cooling rate is between about 0.25°C and 2.0°C per minute.

20 24. A method as in claim 21 wherein said tissue is human kidney
slices and said cooling rate is between about 8°C and 15°C per minute.

25 25. A method as in claim 13 wherein said cryoprotectant comprises
from about 5% to about 50% by weight of said solution.

26. A method as in claim 13 wherein said contacting step comprises
sequentially placing the tissue in a series of solutions, each solution in the sequence
having a greater concentration of cryoprotectant than the previous solution.

30 27. The method of claim 19 further comprising the step of thawing
the cryopreserved human tissue slices.

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28. The method of claim 27 wherein the cryoprotectant comprises
1 liquid nitrogen and said thawing step comprises:
warming the tissue to between about -150°C and about -100°C
by raising the tissue out of the liquid nitrogen and into the gas phase above the liquid
5 nitrogen;
warming the tissue slices to between about -70°C and about
-50°C by raising the tissue further above the liquid nitrogen and higher into the gas
phase; and
10 warming the tissue slices to 37°C by immersing the slices into a
large volume of said preservation solution which has been heated to 37°C.
29. A method as in claim 27 wherein said thawing step comprises:
15 placing the slices in a preservation buffer solution for about 2 to
about 24 hours, the solution comprising:
from about 30 to about 120 mM gluconate (Na⁺);
from about 30 to about 120 mM saccharate (Na⁺);
from about 25 to about 30 mM phosphate;
20 from about 1 to about 3 mM sodium succinate;
from about 3 to about 6 mM magnesium sulfate;
from about 1 to about 3 mM magnesium chloride;
from about 1 to about 3 mM sodium bicarbonate;
from about 2 to about 4 mM glucose;
25 from about 2 to about 4 mM fructose;
from about 3 to about 6 mM glutathione;
from about 2 to about 10 mM ascorbic acid (Na⁺);
from about 3 to about 5 mM adenosine; and
30 micromolar amounts of antibiotics and deferoxamine mesylate.

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30. A method as in claim 28, further comprising the step of
1 contacting the tissue with a final culturing medium at a temperature of up to 38°C for
up to 72 hours, the final culture medium being selected from the group consisting of
fetal calf serum or said preservation buffer solution.

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31. A method of packaging a tissue slice comprising:
affixing the tissue slice to a screen by: a) contacting the tissue
slice with a solution comprising a carrier material in a solvent; and b) placing the
10 solution covered tissue slice onto the screen; whereby the carrier material forms a gas
and liquid permeable film and affixes the slice to the screen.

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32. A container comprising:
an inner container having a spherical inner surface;
an outer container having a recess for receiving said inner
16 container;
a self-righting member within said inner container, said member
having a riding surface which is substantially parallel to said inner surface of said
20 inner container; and
a lubricating material between said member and said inner
container.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10650

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : IPC(5): A01N 1/02; C12N 5/00, 1/04

US CL : 435/1, 2, 240.1, 240.2, 260; 220/903; 252/176

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1, 2, 240.1, 240.2, 260; 220/903; 252/176

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS, BIOSIS, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,980,277 (JUNILLA) 25 DECEMBER 1990, SEE ENTIRE DOCUMENT.	1-32
Y	US, A, 4,920,044 (BRETAN, JR) 24 APRIL 1990, SEE ENTIRE DOCUMENT.	1-32
Y	US, A, 4,609,622 (KOHN ET AL) 02 SEPTEMBER 1986, SEE ENTIRE DOCUMENT.	1-32
Y	TRANSPLANTATION, VOLUME 45, NUMBER 4, ISSUED APRIL 1988, BELZER ET AL, "PRINCIPLES OF SOLID-ORGAN PRESERVATION BY COLD STORAGE," PAGES 673-676, SEE ENTIRE DOCUMENT.	1-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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* P	* A	document published prior to the international filing date but later than the priority date claimed
		document member of the same patent family

Date of the actual completion of the international search

19 February 1993

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